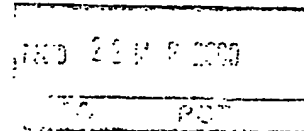


PCT/GB 00/00238
03 March 2000

GB 00/238



CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 7 October 1999 with an application for Letters Patent number 500190 made by UNIVERSITY COLLEGE LONDON.

Dated 19 January 2000.

Neville Harris
Commissioner of Patents

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17 I(a) OR (b)

BEST AVAILABLE COPY

500 190

Patents Form No. 4

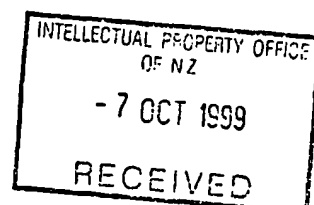
PATENTS ACT 1953

PROVISIONAL SPECIFICATION

FORMULATIONS

We, **UNIVERSITY COLLEGE LONDON**, an institute incorporated by Royal Charter of Gower Street, London WC1E 6BT, United Kingdom do hereby declare this invention to be described in the following statement:

-1-
(followed by page 1A)



FORMULATIONS

This invention relates to formulations for use in therapeutic and/or cosmetic treatments, particularly those in which a localised disruption in direct cell-cell communication is desirable.

BACKGROUND

Gap junctions are cell membrane structures which facilitate direct cell-cell communication. A gap junction channel is formed of two hemichannels (connexons), each composed of six connexin subunits. These connexins are a family of proteins, commonly named according to their molecular weight or classified on a phylogenetic basis ie. into an α class and a β class.

An ability to control connexin expression (and in particular to downregulate it) would therefore provide an opportunity to modulate cell-cell communication within a patient for therapeutic and/or remedial purposes. However, as a number of connexin proteins are expressed widely throughout the body, a general downregulatory effect is undesirable in inducing a therapeutic effect at a specific site.

Anti-sense oligodeoxynucleotides (ODN's) have considerable potential as agents for the manipulation of specific gene expression (reviewed. Stein *et al.*, 1992; Wagner 1994). However, there remain difficulties which need to be overcome. These include the short half life of such ODN's (unmodified phosphodiester oligomers typically have an intracellular half life of only 20 minutes owing to intracellular nuclease degradation (Wagner 1994)) and their delivery consistently and reliably to target tissues.

It was with the intent of at least partially overcoming these difficulties that the applicants devised the present invention.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect, the invention provides a formulation for use in therapeutic and/or cosmetic treatment, which formulation comprises:

at least one anti-sense oligodeoxynucleotide to a connexin protein; and

a sustained release vehicle for said oligodeoxynucleotides.

5

In one preferred form, the formulation contains oligodeoxynucleotides to one connexin protein only. Most preferably, this connexin protein is connexin 43.

Alternatively, the formulation contains oligodeoxynucleotides to more than one
10 connexin protein. Preferably, one of the connexin proteins to which oligodeoxynucleotides are directed is connexin 43. Other connexin proteins to which oligodeoxynucleotides are directed include connexin 26, connexin 31.1 and connexin 32.

15 Conveniently, the oligodeoxynucleotide to connexin 43 is selected from:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC;
GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC; and
GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT

20

Most conveniently, the oligodeoxynucleotide to connexin 43 is:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC.

25 Conveniently, the oligodeoxynucleotide to connexin 26 is:

TCC TGA GCA ATA CCT AAC GAA CAA ATA.

Conveniently, the oligodeoxynucleotide to connexin 31.1 is:

30

CGT CCG AGC CCA GAA AGA TGA GGT C.

Conveniently, the oligodeoxynucleotide to connexin 32 is:

35

TTT CTT TTC TAT GTG CTG TTG GTG A.

Preferably, the sustained release vehicle is, or includes, a gel.

Conveniently, the gel is a Pluronic gel, most preferably Pluronic F-127.

- 5 Conveniently, the formulation further includes a surfactant to assist with oligodeoxynucleotide cell penetration.

In a further aspect, the invention provides a method of site-specific downregulation of connexin protein expression for a therapeutic and/or cosmetic purpose which
10 comprises administering a formulation as defined above to a site on or within a patient at which said downregulation is required.

In still a further aspect, the invention provides a method of reducing neuronal cell death which would otherwise result from a neuronal insult to a specific site in the
15 brain, spinal cord or optic nerve of a patient which comprises the step of administering a formulation as defined above to said site to downregulate expression of connexin protein(s) at and immediately adjacent said site.

Preferably, the formulation is administered to reduce neuronal loss due to physical
20 trauma to the brain, spinal cord or optic nerve.

Conveniently, the formulation is administered in a sufficient amount to downregulate expression of said connexin protein(s) for at least 24 hours post-administration.

25 In yet a further aspect, the invention provides a method of promoting wound healing in a patient which comprises the step of administering a formulation as defined above to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

30 Usually, the wound will be the result of trauma, including burns. It may however be the result of surgery.

In yet a further aspect, the invention provides a method of reducing inflammation as
35 part of treating a wound and/or tissue subjected to physical trauma which

comprises the step of administering a formulation as defined above to or proximate to said wound or tissue.

Preferably, said wound is a burn.

5

Alternatively, said wound is the result of physical trauma to tissue, including neuronal tissue such as the brain, spinal cord or optic nerve.

10

In yet a further aspect, the invention provides a method of decreasing scar formation in a patient who has suffered a wound which comprises the step of administering a formulation as defined above to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

15

Again, the wound may be the result of trauma or surgery, with the formulation being applied to the wound immediately prior to surgical repair and/or closure thereof.

20

In yet a further aspect, the invention provides a method of skin rejuvenation or thickening for a cosmetic or therapeutic purpose which comprises the step of administering, once or repeatedly, a formulation as defined above to the skin surface.

25

Conveniently, said formulation includes oligodeoxynucleotides directed to connexin 26 or connexin 43 and is administered to regulate epithelial basal cell division and growth.

30

In another embodiment, said formulation includes oligodeoxynucleotides directed to connexin 31.1 and is administered to regulate outer layer keratinisation.

Preferably, the formulation is a cream.

DESCRIPTION OF THE DRAWINGS

35

Figures 1 to 5 show sections of rat brain lesions treated with Pluronic gel containing antisense oligodeoxynucleotides specific to connexin 43, or for control lesions,

Pluronic gel alone. In all cases lesions were sectioned serially in a coronal plane and the mid point sections used for analysis. Each image (except Figure 5) shows 4 mm by 5.33 mm of tissue. Figure 5 is approximately 1.2 mm by 2 mm.

5 Figure 1: Figures 1A and 1C show two side of a control lesion 24 hours after lesioning. The lesion has been treated with Pluronic gel alone. The sections have been Nissl stained (blue nuclei) and antibody labelled with the neuronal marker Neuronal-N (brown cells). Figures 1B and 1D show grey scale images of 1A and 1C respectively with the outline of the lesion marked. Note the large size of the lesion and the irregular spreading edges. The lesion has spread downwards toward the corpus callosum (dashed line) within just 24 hours of lesioning.

15 Figure 2: A control lesion 24 hours after wounding. Figure 2A shows Nissl staining (blue nuclei) and Neuronal-N labelling of viable neurons. Figure 2B is a grey scale equivalent with the lesion edge marked and the top of the corpus callosum marked (dashed line). The original needle tract is clear but neuronal death has occurred well back from the lesion edge as indicated by the Neuronal-N labelling. The edges of the lesion are irregular and the lesion, within just 24 hours, has spread right down into the corpus callosum.

20 Figure 3: Figures 3A and 3B are colour and grey scale images of a connexin 43 antisense treated lesion, 48 hours after lesioning. The lesion outline has been marked on Figure 3B to show the extent of the lesion and the top of the corpus callosum marked (dashed line). Figure 3A has been stained for Nissl (blue nuclei) and Neuronal-N (pink cells). Note how compact the lesion is, even after 48 hours, compared with control lesions (Figures 1 and 2). While there is some spread to the right hand side, the left side of the lesion essentially follows the original needle tract with little sign of spreading. The left side of the lesion is very straight and it has not spread down to the corpus callosum.

30 Figure 4: Figures 4A and 4B show another connexin 43 antisense treated lesion 48 hours after wounding. The labelling is the same as in Figure 3 with the lesion outlined on the grey scale image (Figure 4B). Even after 48 hours this lesion is extremely compact with slight spreading only to the left (medial side). Note how straight the right hand side of the lesion is with viable neurons right up to the edge

of the needle tract (and indeed surviving within the lesioned area). The lesion is well above the corpus callosum (dashed line) indicating virtually no downward spread.

Figure 5: A higher magnification view showing the edge of a connexin 43 antisense treated lesion. The edge of the lesion has been marked showing viable neurons (Neuronal-N labelled) right up to the edge of the wounding needle tract even 48 hours after lesioning.

Figure 6: GFAP (red) and connexin43 (green) immunohistochemical labelling of a connexin43 specific antisense treated lesion, 24 hours after lesioning. The image is taken at the lateral edge of the lesion at a point half way down the depth of the lesion. Activated astrocyte levels are elevated compared with controls (Figure 7) and connexin 43 levels are markedly reduced. The connexin labelling remaining is generally associated with blood vessels (arrows).

Figure 7: GFAP (red) and connexin43 (green) immunohistochemical labelling of a control lesion, 24 hours after lesioning. The image is from the medial edge of the lesion and shows GFAP levels slightly elevated over unlesioned cortex. Note the extensive connexin 43 labelling, often co-localised with the GFAP astrocytic marker (arrows).

Figure 8 shows a comparison of lesion cross sectional lower half areas 24 hours (circles) and 48 hours (diamonds) after lesioning. The analysis was carried out on a mid section of serially sectioned lesion cut on the coronal plane. Lesions were assessed using Neuronal-N antibody labelling to delineate viable neurons. DB1 treated lesions (green markers) have been treated with antisense oligodeoxynucleotides specific to connexin 43. The gel only lesion group (red markers) also includes empty lesions while the HB3 group (purple markers) are treated with gel containing random sequence control oligodeoxynucleotides. Note that while connexin 43 antisense treated lesions can be large (presumably where the antisense has not been well delivered), the smallest lesions are all connexin 43 antisense treated. Lesions were made to a depth of 2 mm and analysis covers 1 mm and below so as to exclude the outer edge where the antisense did not sit.

Figure 9: Lesions in rat spinal cord 24 hours after treatment with connexin 43 sense and antisense ODN's. The sense lesions were no different from untreated

controls whereas the antisense treated lesions were smaller and with reduced inflammation.

Figure 10: Lesions in neonatal mouse fore paws 24 hours after treatment with connexin 43 sense ODNs (left paw) or antisense ODNs (right paw). Note the reduction in inflammation and increased rate of healing on the antisense treated paw.

Figure 11: Sections through the centre of the 24 hour wounds shown in Figure 10. The sections have been stained with toluidine blue to reveal neutrophils. There are significantly less neutrophils in the antisense treated wound which was also less inflamed.

Figure 12: Pairs of rat paw lesions five days after lesioning that have been treated with connexin 43 specific antisense ODNs or sense control ODNs. Antisense treated lesions are healing quicker and show less signs of scarring.

Figure 13: Pairs of rat paw lesions made at the neonate stage, and viewed here 8 days after lesioning. Lesions were treated with connexin 43 specific antisense or control sense ODN. Hair has grown and it is clear that antisense treatment has resulted in smaller scars and less hair loss. The site of the lesion remains prominent in the sense treated control but is difficult to detect in the antisense treated limb

25 DESCRIPTION OF THE INVENTION

As broadly defined above, the focus of the invention is on site-specific downregulation of connexin expression. This will have the effect of reducing direct cell-cell communication at the site at which connexin expression is downregulated, which gives rise to numerous therapeutic/cosmetic applications as described below.

The downregulation of connexin expression is based generally upon the anti-sense approach, and more particularly upon the use of anti-sense oligodeoxynucleotides (ODN). These ODN target the connexin protein(s) to be downregulated.

35

The connexin protein or proteins targeted by the ODN will be dependent upon the site at which downregulation is to be effected. This reflects the non-uniform make-up of gap junction(s) at different sites throughout the body in terms of connexin sub-unit composition.

5

Some connexin proteins are however more ubiquitous than others in terms of distribution in tissue. One of the most widespread is connexin 43. ODN's targeted to connexin 43 are therefore particularly suitable for use in the present invention.

10 It is also contemplated that ODN's targeted at separate connexin proteins be used in combination. For example, ODN's targeted to connexin 43, and one or more other members of the connexin family (such as connexin 26, 31.1, 32, 40 and 45) can be used in combination.

15 The ODN's for use in the invention will generally be unmodified phosphodiester oligomers. They will vary in length but with a 30 mer ODN being particularly suitable.

The precise sequence of the ODN used in the invention will depend upon the target
20 connexin protein. For connexin 43, the applicant's have found ODN's having the following sequences to be particularly suitable:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC;
GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC; and
25 GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT

ODN's directed to other connexin proteins can be selected in terms of their nucleotide sequence by any convenient, and conventional, approach. For example, the computer programmes MacVector and OligoTech (from Oligos etc. Eugene,
30 Oregon, USA) can be used. For example, ODN's for connexins 26, 31.1 and 32 have the following sequences:

5' TCC TGA GCA ATA CCT AAC GAA CAA ATA (connexin 26)
5' CGT CCG AGC CCA GAA AGA TGA GGT C (connexin 31.1)
35 5' TTT CTT TTC TAT GTG CTG TTG GTG A (connexin 32)

Once selected, the ODN's can be synthesised using a DNA synthesiser.

For use in the invention, the ODN(s) require site-specific delivery. They also require delivery over an extended period of time. While clearly the delivery period will be dependent upon both the site at which the downregulation is to be induced and the therapeutic effect which is desired, continuous delivery for 24 hours or longer will often be required.

In accordance with the present invention, this is achieved by inclusion of the ODN(s) in a treatment formulation which comprises the ODN(s) and a sustained release vehicle. While any such vehicle which is non-toxic may be employed (depending upon the desired ODN release profile), it is preferred that a gel, gel-based or cream-based vehicle be used.

Pluronic-type gels have been found to be particularly suitable. The presently most preferred vehicle is the gel Pluronic F-127 (BASF Corp). This gel is preferred as it is liquid at low temperatures but rapidly sets at physiological temperatures, which confines the release of the ODN component it contains to the site of application or immediately adjacent that site.

20

In addition to the ODN and sustained release vehicle, the formulation may also contain a surfactant, but may include any loading reagent. The purpose of including a surfactant is to assist the ODN component in cell penetration.

Where a surfactant component is included, any conventional non-toxic surfactant can be selected. An example is DMSO.

Once prepared, the formulations of the invention have utility in any therapeutic/cosmetic approach where a transient and site-specific interruption in cell-cell communication is desirable. These include in treating neuronal damage in the brain, spinal cord or optic nerve (where the damage is to be localised as much as possible), in the promotion of wound healing and in reducing scar formation following, for example, cosmetic surgery or burns.

In particular, topical formulations such as creams can be employed to regulate epithelial basal cell division and growth (using ODN's targeted to connexin 43) and outer layer keratinisation (using ODN's targeted to connexin 31.1).

- 5 Various aspects of the invention will now be described with reference to the following experimental section which will be understood to be provided by way of illustration only and not to constitute a limitation on the scope of the invention.

EXPERIMENTAL

10

EXPERIMENT 1

MATERIALS AND METHODS

15 Antisense application

- 30% Pluronic F-127 gel (BASF Corp) in phosphate buffered saline (molecular grade water) was used to deliver unmodified $\alpha 1$ connexin (connexin 43) specific anti-sense ODN's to the developing chick embryo (Simons, *et al.*, 1992). Chick embryos were incubated at 38°C and staged according to Hamilton and Hamburger stages. Eggs
20 were windowed and the vitelline and amniotic membranes over the area to be treated were opened using fine forceps. After anti-sense application eggs were sealed with tape and replaced in the incubator for 48 hours at which time most experiments were analysed, the exception being for the time course analysis of $\alpha 1$ connexin "knockdown" and recovery.

25

- Pluronic gel is liquid at low temperatures, 0-4°C, but sets when dropped onto the embryo at physiological temperature, remaining in place for at least 12 hours. The gel has the additional advantage of being a mild surfactant and this, used either alone or in conjunction with DMSO, appeared to markedly expedite ODN
30 penetration into cells (Wagner, 1994). Addition of an FITC tag to DB1 ODN, viewed using confocal laser scanning microscopy, demonstrated intracellular penetration of the probes. Sequences of deoxyoligonucleotides used are shown in Table 1.

Table 1: The Effect on Limb Development of ODN Application Between Stages 8 & 14 of Chick Embryo Development

Antisense oligodeoxynucleotides to Connexin 43
 DB1 GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC
 CG1 GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT
 Control oligodeoxynucleotides
 DB1(sense) GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC
 DB1(chick) GTA GTT ACG ACA GGA GGA ATT GTT CTC GTC
 CV3(random) TCG AAC TGT CAA GAC TGC TAT GGC GAT CAT
 Gel only

5

All ODN's were applied at 0.5-1.0 μ M final concentration following dose dependent analysis during preliminary experiments covering a range of concentrations from 0.05 μ M to 50 μ M. General toxicity effects only became apparent with ODN concentrations greater than 10 μ M. ODN gel mixtures were prepared from concentrated stock solutions stored at -80°C.

10

Anti-sense sequences

DB1 is a mouse anti-sense sequence, complementary to bases 1094 - 1123 of the α 1 connexin gene. It has four mismatches with chick α 1 connexin sequence. CG1 is complementary to chick α 1 connexin bases 720-749. Efficacy of this probe was improved with 1% Dimethylsulphoxide (DMSO) added to the gel. DMSO had no added effect on other anti-sense ODN or control results.

15

Control sequences

DB1(Chick) is the chick α 1 connexin equivalent of DB1 matching chick α 1 connexin bases 954-983. Analysis however, indicates a high probability of forming stem loop structures ($G = -7.0$ kcal/mol, Loop $T_m = 92^\circ$) and homodimerisation ($T_m = 1.5^\circ$) and therefore acts as a control sequence. It has been reported that some sense oligonucleotides can form stable DNA triplets (Neckers *et al.*, 1993) inhibiting transcription. However, this was not apparent with DB1 (sense). A random control sequence with no stable secondary structure ($G = 1.4$ kcal/mol) and unstable homodimerisation was also used, called CV3. An additional control applying equal concentration mixture of DB1 and DB1 (sense) gave background levels of defects.

25

Monitoring of protein knockdown

Immunohistochemical localisation of $\alpha 1$ connexin gap junction protein at cell-cell interfaces provides a direct measurement of the anti-sense effect. Anti-peptide $\alpha 1$ connexin specific antibody probes were used to stain wholemount embryos and the connexin distribution was analysed using confocal laser scanning microscopy according to established procedures (Green *et al.*, 1995). Control labelling for two other connexins expressed in the developing chick embryo (connexins $\beta 1$ & $\beta 2$) was similarly carried out, also using sequence specific antibodies (Becker *et al.*, 1995).

10 RESULTS

Reduction of $\alpha 1$ connexin expression

Using Pluronic F-127 gel to deliver unmodified $\alpha 1$ connexin specific anti-sense ODN's to the developing chick embryo, protein expression can be interfered with at chosen time points and allows the anti-sense treatment to be targeted to specific regions of a chick embryo. A droplet of gel containing the anti-sense at a relatively low concentration was placed precisely onto individual embryos. The gel sets and remains in place for at least 12 hours and thus a sustained low dose of anti-sense is maintained in this region. The anti-sense applications were targeted and timed to block junction formation prior to the periods of elevated expression in the limb, neural tube and face. These times were chosen to optimise the effects of the anti-sense by reducing the expression of new protein rather than being dependent upon the turnover of protein already in the membranes of the cells of the target tissue. Both DB1 and CG1 ODN's reduced expression of $\alpha 1$ connexin protein within two hours in the neural tube and limb bud, dramatic within 4-8 hours and persisted at 18-24 hours and 48 hours in some tissues (data not shown). No down regulation of $\alpha 1$ connexin protein was evident in any of the controls used. Equally, two other members of the connexin family expressed in the chick embryo, $\beta 1$ connexin and $\beta 2$ connexin, were unaffected by the $\alpha 1$ connexin specific anti-sense ODN.

Several parallel controls were run with all of the experiments. These included; DB1 sense, DB1 anti-sense and DB1 sense combined, DB1 chick (which forms stem loop structures with itself), random ODN's CV3, Pluronic gel alone, Pluronic gel with DMSO and PBS alone). None of the controls had a noticeable effect on $\alpha 1$ connexin protein expression.

EXPERIMENT 2

INTRODUCTION

5 Astrocytes constitute the most abundant cell type in the mammalian brain. They are extensively coupled to one another and to neurons through gap junctions composed predominantly of connexin 43 (Giaume and McCarthy (1996)). Following ischaemia induced or physical brain damage these channels remain open and a spreading wave of depression (initiated by raised interstitial potassium and glutamate and apoptotic signals) is propagated (Cotrina *et al.*, (1998); Lin *et al* (1998)). Waves of increased cytosolic calcium and second messenger molecules such as IP3 are slowly spread via the gap junction channels to neurons beyond the core of the damaged region, resulting in lesion spread in the 24-48 hours following the insult. In this manner, undamaged neighbouring cells are destroyed (Lin *et al.*, 15 1998), the so-called bystander effect.

This experiment investigates the ability of the formulations of the invention to prevent this bystander effect.

20 MATERIALS

Oligodeoxynucleotides were prepared with the following sequences:

25 GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (connexin 43)
TTG TGA TTT ATT TAG TTC GTC TGA TTT C (random control)

METHODS

Oligodeoxynucleotides (ODN's)

30 Unmodified ODN's were delivered in Pluronic F-127 gel (BASF) in phosphate buffered saline (PBS). Pluronic gel is liquid at low temperatures (0-4°C) and sets at physiological temperatures, and is also a mild surfactant. Unmodified ODN's normally have a half life of approximately 20 min in cells (Wagner, 1994) but the Pluronic gel loading method provides a continual diffusion source, the gel acting as a reservoir (Becker *et al.*, (1999)). ODN's specific to connexin 43 were applied, or 35 control random ODN's of similar base composition, at 2µM final concentration. Gel

only controls were also carried out. ODN's were 30 mers analysed to show that no hairpin looping or homodimerisation should occur.

Lesioning

- 5 Brain lesions were carried out on 250-300 g male Wistar rats. Animals were anaesthetised with 1-2% halothane in oxygen and the head held in a stereotaxic clamp. The region around the lesion site was shaved and the skin over the skull slit in a sagittal plane with a scalpel and pulled back to leave the skull plates clear. A 0.5 mm diameter hole was drilled through the skull plate 3 mm to the right of
- 10 bregma using an Arlec engraver and a lesion made into the cortex of the brain using a 19G 1½ gauge syringe needle attached to a micrometer stage. The stage allowed accurate directional control and a precise 2 mm penetration depth which kept the lesion within the cortex and well above the corpus callosum.
- 15 With the animal prepared, 10µl of ice cold Pluronic F-127 gel (BASF) containing connexin 43 specific ODN (or a control ODN) was sucked into a precooled 19 G 1½ gauge syringe needle filed off so as to have a flat tip. The syringe needle was attached to a volumetric pipette via a cut down yellow pipette tip. The gel then set in the needle as it warmed to room temperature. The needle with the gel plug at its
- 20 tip was transferred to a 1 ml syringe containing PBS and a sleeve slipped over the needle shaft so that the needle tip could be lowered into the lesion with the sleeve (coming up against the skull) preventing overpenetration. Gentle pressure on the syringe plunger "popped" the gel plug out of the needle into the lesion. The wound was then treated with hydrogen peroxide to stop bleeding and the skin sutured back
- 25 into place. Animals were carefully monitored and left until ready for sacrifice 24 hours, 48 hours or 12 days later.

Frozen Sectioning

- 30 Animals were sacrificed using Nembutal (pentobarbitone sodium, Virbac) and decapitated. The brains were removed intact and immediately frozen in dry ice snow and stored at -80°C until ready for sectioning. Serial cryosections (30 µm sections) were taken from front to rear (coronal plane), the sections dry mounted onto chrome alum treated slides, and stored for histochemistry or immunohistochemistry at -80°C. The first and last section of each lesion was
- 35 recorded so that the mid-point sections of the lesion were clearly identified.

Histochemistry

For haematoxylin and eosin staining sections were hydrated through a descending series of alcohols (absolute, 2 x 95%, 1 x 70% and water) and stained in Gill's haematoxylin for 4 minutes. The sections were then washed in water, dipped in Scott's water and rewashed in water. They were then stained for 30 seconds in Moore's buffered eosin. The sections were washed once more in water before dehydration through a series of alcohols (2 x 95%, 1 x absolute), 50:50 alcohol:xylol and dipped in xylene. The sections were then mounted using Histomount™ mounting medium.

10

For Nissl staining, sections were dehydrated in an ascending graded series of alcohols (75%, 95%, 3 x 100%), five minutes in each, and defatted in xylene for five minutes. The sections were then rehydrated by descending through the same series of alcohols and washed in water. The sections were then placed in a Nissl staining solution (5 ml of a 2% aqueous Cresyl violet stock solution, 90 ml of a 6% glacial acetic acid in water solution, 10 ml of a 1.35% sodium acetate solution) for 10 minutes. The sections were then quickly dehydrated in a series of ascending alcohols for 5 minutes at 75%, then 2 minutes each at 95% and 3 x 100%, three charges of xylene for 10 minutes each. They were then coverslipped with Histomount™ mounting medium.

20

Immunohistochemistry

Frozen sections were first allowed to come back up to room temperature in PBS. They were then permeabilised in methanol for two minutes, rinsed in PBS and transferred to a solution of 0.1M lysine and 0.1% Triton-X 100 in PBS for blocking over 30 min. Two washes in PBS, each of two minutes, followed. PBS was removed and 50 µl per section of primary antibody was applied.

25

Immunohistochemistry was carried out with primary antibodies against connexin 43, Neuronal-Nuclei (vertebrate specific nuclear protein NeuN) and GFAP (glial fibrillary acidic protein). The following antibodies were used:

30

Rabbit anti-Cx 43 (Gourdie *et al.*, (1991)) at a concentration of 1:300.

Mouse anti-Cx 43 (Chemicon International, Inc.) at a concentration of 1:100.

35

Rabbit anti-rat GFAP (DAKO, Z0334), at a concentration of 1:1000.

Mouse anti-Neuronal Nuclei (Chemicon International, Inc.) at a concentration 1:1000.

For connexin and GFAP labelling sections were incubated overnight at 4°C. They
 5 were then washed three times 15 minutes in PBS on an orbital shaker. Following
 this, excess PBS was removed and 50 µl per section of Alexa™ 488 anti-rabbit IgG
 (Molecular Probes, Oregon, USA) was applied at a concentration of 1:200. For
 monoclonals and double labelling a CY3 (Chemicon, 132C) anti-mouse secondary
 antibody was used. Sections were incubated in the dark for two hours at room
 10 temperature followed by three washes of 15 minutes in PBS. For mounting excess
 PBS was removed from the slides and one or two drops of Citifluor (glycerol/PBS
 solution) anti-fade medium was applied. A coverslip was lowered onto the sections
 and sealed with nail varnish. For Neuronal-N labelling the secondary antibody was
 a biotinylated Goat anti-mouse followed by an avidin linked HRP and DAB reaction
 15 (Sigma ExtrAvidin or DAKO Quickstain kit).

Imaging and Analysis

Immunofluorescent labelling was carried out using a Leica TCS 4D confocal laser
 scanning microscope. Double labelled images were subsequently combined using
 20 the Leica Combine function or in Adobe Photoshop. Haemotoxylin and eosin, and
 Nissl stained samples or Neuronal-N labelled sections were captured using a
 Kontron (Zeiss) Progress 3008 digital camera and lesion areas analysed using
 MetaMorph (Universal Imaging Corp). Lesion areas were analysed for the middle
 section of each lesion.

25

RESULTS

The well documented spread of brain lesions in the first 24 -48 hours after trauma
 occurred in our control gel experiments and all lesions, controls and antisense
 30 treated, tended to spread near the outer edge where the gel is less likely to sit after
 loading. However, control lesions spread downwards into the corpus callosum and
 sideways to form ragged, spreading edges (Figures 1 and 2). Examination of
 Neuronal-N antibody labelled tissues reveals neuronal death occurring well back
 from the lesion edge, with areas of Nissl staining in which no viable neurons remain.
 35 This spread occurs predominantly within 24 hours (Figures 1 and 2), continuing up
 to 48 hours after lesioning. This is especially apparent in Figure 2 where neuronal

death is evident within 24 hours well back from the lesion edge into otherwise normal looking tissue, and the lesion has spread right down into the corpus callosum. In contrast, the better connexin 43 antisense treated lesions remain confined to the original lesion site and have clearly defined base levels (Figures 3 and 4). Neuronal-N labelling colocalises with Nissl stained tissue and none of the connexin 43 antisense treated lesions spread through the corpus callosum. Neuronal-N labelling shows neuronal survival right up to the edge of the original needle tract lesion. Surviving neurons around these lesions often define sharp boundaries marking the edge of the needle tract (Figures 3 and 5). More tissue remains viable within the lesion itself after antisense treatment; in control lesions cell death leads to tissue loss within the lesion area (compare control lesion in Figure 2 at 24 hours with antisense treated lesions in Figures 3 and 4 at 48 hours).

While antibody labelling of glial fibrillary acidic protein (GFAP) shows some increased astrocyte activation at the edges of lesions, connexin 43 protein levels are clearly reduced at many places along the edge of antisense treated lesions, particularly the basal and medial edges (Figure 6) compared with controls (Figure 7). In some areas the only connexin 43 labelling remaining 24 hours after connexin 43 specific antisense treatment is in blood vessel walls despite raised GFAP levels (Figure 6). In general, connexin 43 labelling around antisense treated lesion colocalises to a much lesser extent with GFAP labelling than in controls in which over half of the connexin 43 labelling is astrocyte related. Other connexin levels (connexins 26 and 32) did not appear to be altered by the connexin 43 specific antisense treatments.

25

36 animals were lesioned. Cross sectional area (central slice of the lesion volume in a coronal plane) was analysed for 21 animals. The results are shown in Table 2.

Table 2

Cross sectional areas of lesions treated with control and connexin 43 specific oligodeoxynucleotides, left empty, or treated with gel only. Measurements are for animals measured after 24 hours, 48 hours and 12 days. Two sets of figures are included - measurements of the entire lesion, and measurements from 1 mm below the surface. In analysis of the second group the largest DB1 treated lesion (brackets) is excluded as it falls outside 3 standard deviations from the mean for this group. Note that the rat brain does heal (unlike other species) and 12 days lesion measurements do not represent the original extend of lesion spread.

DB1 is anti connexin 43 treated
HB3 is random oligo and appears to be toxic

Entire Lesion: (measurements in square mm)

	24 hours	48 hours	12 days
DB1	2.42; 3.16; 3.78; 5.57	3.7; 6.05; 2.91; 3.41; 4.53	2.79; 2.86
HB3	7.14	13.19	
Gel/empty	5.04; 4.48	3.96; 3.41; 3.56; 5.91	2.58; 3.3

Lesions from 1 mm down: (this is considered a more accurate measure as all lesions tend to spread at the outer lip indicating that the treatment gel has settled in the bottom of the lesion and/or the outer cortex has been damaged when drilling the skull or inserting the gel loading needle).

	24 hours	48 hours	12 days
DB1	0.91; 1.13; 2.12; 2.41	(3.38); 0.99; 1.54; 1.44; 1.08	0.47; 1.2
HB3	5.9	5.6	
Gel/empty	3.2; 2.19	1.86; 1.5; 1.68; 2.17	1.07; 1.43

In the final analysis the lesion area from a line 1 mm below the outer cortex edge was measured so as to exclude lesion spread at the outer edge where antisense treatments have little or no effect (owing to gel being injected into and settling at the bottom of lesions). One antisense treated animal falls more than three standard deviations outside the mean for this group and has been excluded. Mean lesion size for antisense treated lesions at 24 and 48 hours was 1.45 mm² (+/- 0.55), for controls 2.1 mm² (+/- 0.6). The four smallest (of 8 antisense treated and 8 control lesions at 24 and 48 hours) were all connexin 43 antisense treated, with the smallest control lesion 50% larger than these four. This data is also shown in graphical form in Figure 8. By 12 days regeneration occurs in the rat (but not in human brain tissue) and the limits of lesion spread are not clearly defined.

DISCUSSION

The Pluronic gel plug - antisense ODN method has been used to study the effect of connexin 43 knockdown during astrocytosis which occurs following lesioning of the cerebral cortex of the mammalian brain. In the brain, release of toxins from dying neurons causes what is known as the bystander effect, with the toxins spreading to neighbouring cells through gap junction channels (Lin *et al*, (1998)). Under neurodegenerative conditions, slow release of toxins apparently leads to an upregulation of connexin 43 channels in astrocytes to enable the transport and removal of the toxins to the blood stream. In cases of severe trauma however, this upregulation aids the spread of high toxin levels to neighbouring neurons, killing them. Blocking of the connexin 43 upregulation and knockdown of connexin 43 channels prevents this spread leading to lesions up to 50% smaller in cross sectional area. This has significant implications in the management of ischemic stroke, treatment of neurodegenerative diseases, and modulation of side effects from surgical intervention.

EXPERIMENT 3

20 INTRODUCTION

The bystander effect in neural tissues whereby damaged neurons release toxins which spread and kill neighbouring cells is well documented. Experiment 2 shows that this effect can be reduced in the brain using an antisense oligodeoxynucleotide sustained release approach to knockdown the gap junction protein connexin 43.

Another tissue of similar composition to the brain is the spinal cord in which the neural population is supported by populations of glial cells, including astrocytes which are responsible for the neuroprotective effect by removing glutamate and excess calcium from the neural environment. This experiment investigates the ability of the formulations of the invention to reduce the spread of spinal cord lesions.

MATERIALS

Oligodeoxynucleotides were prepared with the following sequences:

- 5 GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (connexin 43)
 GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC (sense control)

METHODS

- 10 Wistar rats were anaesthetised and their spinal cord exposed. A standard hemisection lesion was then made in the cord and 5 μ l of chilled Pluronic gel, containing either antisense or sense ODN's to connexin 43 (5 μ M) was placed in the lesion. Applications were made blind. The exposed cord was then recovered and the rat returned to its cage. Some animals were sacrificed at 24 hours whereas
- 15 others were maintained for 12 days and two months in order to determine the extent of neuronal regeneration and the final size of the lesion. For axonal regeneration studies the rats were anaesthetised and their axons severed prior to their entry site to the spinal cord. A pellet of Horse radish peroxidase (HRP) was placed in the cut in order to retrogradely label the axons over a 24 hour period.
- 20 Next day the rats were sacrificed and their spinal cords removed and fixed in 2% paraformaldehyde. Cords were then processed for cryosectioning and serial longitudinal 8 μ m sections were taken through the cords. Sections were then immunostained for either connexins or GFAP along with propidium iodide as a nuclear marker, or processed to reveal the HRP.

25

RESULTS

- At 24 hours post lesion there was a marked difference between the spinal cord lesions treated with connexin 43 sense and antisense ODN's. The sense lesions
- 30 appeared no different from untreated controls whereas the antisense treated lesions appeared smaller and less inflamed (Figure 9).

- At 12 days HRP labelled axons could be seen in both sense and antisense treated cords but in neither case did significant numbers of regenerating axons cross the
- 35 lesion. However, there was a marked difference in lesion size with the antisense lesion appearing significantly smaller than the sense or untreated lesions.

Two months after lesioning the spinal cords HRP labelling of regenerating axons revealed that they had failed to cross the lesion site in both sense and antisense treatments. Lesion size was significantly smaller in antisense treated cords
5 indicating a significant reduction in secondary neuronal cell death.

DISCUSSION

Using the formulations of the invention, the antisense oligodeoxynucleotide
10 knockdown of connexin 43 significantly reduces the lesion spread which occurs in the first 24-48 hours after spinal cord injury. The knockdown of connexin 43 also reduces inflammation, further aiding in the neuroprotective effect, but there was no change in the ability for neurons to grow back across the lesion site. Thus, antisense treatment with connexin 43 specific oligodeoxynucleotides cannot aid
15 regrowth of damaged neurons, but has a significant neuroprotective effect reducing the spread of the insult.

EXPERIMENT 4

20 INTRODUCTION

To repair skin wounds a number of cell types, such as fibroblasts, endothelial cells and keratinocytes are activated to proliferate, migrate and lay down extracellular matrix to fill the wound.

25

Communication and intercellular signalling is a key feature of the wound healing process. Extracellular signalling mechanisms are thought to be the key players though it is also probable that intercellular signalling through the extensive networks of gap junction channels in the skin layers may also have a role. Calcium
30 waves spreading away from injured cells through the epidermis may signal their damage. In normal wound healing connexin levels start to fall within 6 hours and take up to 6 days to recover. The roles that these changes play are not understood but one theory is that cells are released from their neighbours to divide rapidly, and then junctions reform to coordinate migration into and over the wound site.

35

This experiment investigates the ability of the formulations of the invention to effect wound healing.

MATERIALS

5

Oligodeoxynucleotides were prepared with the following sequences:

GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (connexin 43)

GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC (sense control)

10

METHODS

Neonatal mice, CD1 strain, were anaesthetised with local anaesthetic by spray. A clean incision wound, 2 mm long, was then made along the length of both fore paws with an iridectomy knife. By making the wounds under a dissecting microscope they can be made very reproducible in size. They generally heal in 3-6 days. Carbon powder was dusted into the wounds in order to mark them for subsequent identification of the wound site at late time points - this does not affect the healing in any way. 5 µl of chilled Pluronic gel, containing either Sense or Antisense ODN's was then applied to the wounds. The Pluronic gel is liquid between 0-4°C but sets at higher temperature. Once applied to the wound the gel sets in place and acts as a slow release reservoir for the ODN's as well as a mild surfactant, aiding the penetration of ODN's into the tissue. Application of Sense ODN's was made to one paw and Antisense to the other, alternating left and right between litters. Pups were warmed under a lamp and then returned to their mother. Wounds were examined daily and scored for quality of healing. Representative pups were selected at 1 day, 5 day and 8 day post operation and their forelimbs photographed before the pups were anaesthetised and perfused with 2% paraformaldehyde. The forelimbs were removed and immersion-fixed in 2% paraformaldehyde overnight and then processed for resin (1 day) or wax (2 days onward) histology.

Inflammation of the wound was assessed 24 hours after wounding. Resin sections through the wound are stained with Toluidine blue to reveal nissl positive cells, neutrophils, which are the first cells to respond to injury. These can also be revealed using neutrophil specific markers.

Cell death and clearance is assessed by TUNEL labelling to determine the rate of clearance of apoptotic cells. Macrophage staining was used to show the period of clearing up following cell death. These are carried out days 3-5 post wounding.

5 Angiogenesis

Granulation is a feature of healing connective tissue and is caused by the invasion of numerous capillaries. Macrophages are known to express potent angiogenic factors such as VEGF. The degree of vascularisation is monitored with antibodies to VEGF receptors, anti-PCAM and anti- α -1 which are both good blood vessel markers.

- 10 Contraction of this tissue is brought about by the differentiation of wound fibroblasts into a contractile myofibroblast. After they have pulled the wound together they die apoptotically and are removed by macrophages. These cells can be revealed by smooth muscle actin specific antibodies and their formation and removal followed.

15

Hyperinnervation

Sensory nerves are very sensitive to the signals released on wounding and show transient sprouting at the sites of adult wounds. However, in neonatal wounds this sprouting is more profuse and results in permanent hyperinnervation. Whilst it is not clear what these signals are it is likely that they are released from inflammatory macrophages. Hyperinnervation is maximal at 7d post wounding and nerve distribution can be revealed using PGP 9.5 antibody against neurofilaments.

- 25 Scarring is normally assessed weeks or months after closure of the wound. However, a reasonable assessment can be made 12 days after wounding. Sections through the wounds are stained with the collagen stain Picrosirius Red and examined on a confocal microscope to determine the collagen density and orientation at the wound site.

30 RESULTS

1 day

- At 24 hours after wounding marked differences were apparent between the sense and antisense treated limbs. Sense treated wounds looked no different from untreated with a normal spectrum of healing grades and rates (Figure 10).
- 35

Antisense treated limbs were markedly different from the controls, they appeared to be less inflamed and the healing rate was generally faster.

Resin sections of representative limbs stained with a nissl stain revealed
5 significantly less neutrophils cells indicating a less inflamed tissue (Figure 11).

5 days

By days after wounding scabs had started to fall off. At this stage most of the
antisense treated wounds appeared to be smaller than the sense treated with either
10 small scabs or less prominent scarring (Figure 12).

8 days

8 days after wounding the limbs had grown hair. Sense treated wounds were still
visible being demarcated by a lack of hair around the wound site. Antisense treated
15 wounds were mostly invisible being covered by normal hair growth. This difference
in hair growth indicates reduced scarring has occurred in the antisense treated
wounds (Figure 13).

CONCLUSIONS

20 Application of connexin 43 antisense ODN's to a wound has a marked affect on the
healing process. The first noticeable effect is a reduction in the inflammation of the
wounds which is noticeable in sections which show a much lower inflammatory
response in terms of levels of neutrophils. As healing progresses, antisense treated
25 wounds heal faster and with less scarring than control lesions.

This reduction in inflammatory response and subsequent improved healing is
possibly owing to reduced neutrophil communication and to a speeding up of
natural healing processes. The antisense ODN's can reduce connexin expression in
30 4-8 hours so they will not have an effect on the initial signalling of wounding but
play a role in the secondary signalling events. It is interesting to note that
neutrophils which invade in response to the wounding normally express large
amounts of connexin 43. It is also possible that they form gap junctions with other
cells in the wound and communicate with them. Reduction in this form of
35 communication may result in a reduction of secreted factors from the neutrophils
and may reduce cell death in the wound as well as granulation and

hyperinnervation. It is also known that under normal conditions connexin protein levels (connexins 26, 31.1 and 43) are reduced in both the epithelial and subdermal layers of wounds starting within 6 hours, and remaining lowered for up to 6 days. The antisense approach may speed up this initial protein reduction by blocking translational processes as protein removal from the membrane is occurring. Certainly, the effects of connexin 43 knockdown immediately following wounding has marked effects on reducing inflammatory levels and increasing healing rates.

EXPERIMENT 5

INTRODUCTION

The inflammation and secondary cell death that follows burning is of major concern. Victims of severe burns over a high percentage of their body often die one or two days after trauma. This experiment investigates the ability of the formulations of the invention to beneficially affect the burn recovery process.

MATERIALS

Oligodeoxynucleotides were prepared with the following sequences:

GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (connexin 43)

GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC (sense control)

METHODS

Reproducible burns are delivered to moistened skin, and Pluronic gel containing antisense ODN's injected subdermal to the burn. A series of burns were made using a soldering iron to the left and right sides of the skull of six newborn mice. The burns on one side of the head were treated with connexin 43-specific ODN in Pluronic gel and those on the other side with sense control ODN in Pluronic gel.

RESULTS

After 24 hours, all six connexin 43 ODN treated burns showed lower levels of inflammation compared with the control burns. These differences were marked
5 (data not shown).

UTILITY

Thus, in accordance with the invention, there are provided formulations by which
10 cell-cell communication can be downregulated in a transient and site-specific manner. The formulations therefore have application in methods of therapy and in cosmetic treatments.

The delivery of the ODN component of the formulation for an extended period (24
15 hours or longer) is a particular advantage in treating neuronal damage. This is because, in most instances of direct physical neuronal insult, neuronal cell loss extends well beyond the site of actual injury to the surrounding cells. This secondary neuronal cell loss occurs within 24 hours of the original injury and is mediated by junction gap cell-cell communication. Downregulation of connexin
20 protein expression therefore blocks or at least downregulates communication between the cells and minimises secondary neuronal cell damage.

Equally, in instances of other tissue damage (particularly wounds) the formulations of the invention have been found effective in both promoting the wound healing
25 process, reducing inflammation and in minimising scar formation. The formulations therefore have clear benefit in the treatment of wounds, whether the result of external trauma (including burns) or surgical intervention.

It will further be appreciated that the above description is provided by way of
30 example only and that modifications can be made, both in terms of the specific ODN's and sustained release vehicles employed without departing from the scope of the present invention.

REFERENCES

- Becker, D. L., Evans, W. H., Green, C. R., Warner, A. (1995): Functional analysis of amino acid sequences in connexin 43 involved in intercellular communication through gap junctions. *J. Cell Sci.* **108**, 1455-1467.
- Becker, D. L., McGonnell, I., Makarenkova, H. P., Patel, K., Tickle, C., Lorimer, J. and Green, C. R. (1999). Roles for $\alpha 1$ connexin in morphogenesis of chick embryos revealed using a novel antisense approach. *Devel. Genetics*, **24**, 33-42.
- Cotrina, M. L., Kang, J., Lin, J. H-C., Bueno, E., Hansen, T. W., He, L., Lie, Y. and Nedergaard, M. (1998). Astrocytic gap junctions remain open during ischemic conditions. *J. Neurosci.*, **18**, 2520-2537.
- Giaume, C. and McCarthy, K. D. (1996). Control of gap-junctional communication in astrocytic networks. *TINS*, **19**, 319-325.
- Gourdie, R. G., Green, C. R., Severs, N. J. (1991). Gap junction distribution in adult mammalian myocardium revealed by an anti-peptide antibody and laser scanning confocal microscopy. *J. Cell Sci.* **99**: 41-55.
- Green, C. R., Bowles, L., Crawley, A., Tickle C. (1994): Expression of the connexin 43 gap junctional protein in tissues at the tip of the chick limb bud is related to epithelial-mesenchymal interactions that mediate morphogenesis. *Devel. Biol.*, **161**, 12-21.
- Lin, J. H., Weigel, H., Cotrina, M. L., Liu, S., Bueno, E., Hansen, A. J., Hansen, T. W., Goldman, S. and Nedergaard, M. (1998). Gap-junction-mediated propagation and amplification of cell injury. *Nature Neurosci.*, **1**, 431-432.
- Neckers, L., Whitesell, L. (1993): Anti-sense technology: biological utility and practical considerations. *Am. J. Physiol.* **265** (lung cell mol physiol), L1-L12.
- Simons, M., Edelman, E. R., DeKeyser, J. L., Langer, R., Rosenberg, R. D. (1992): Anti-sense c-myc oligonucleotides inhibit intimal arterial smooth muscle cell accumulation *in vivo*. *Nature*, **359**, 67-70.

Stein, C. A. (1992): Anti-sense oligodeoxynucleotides - promises and pitfalls, *Leukemia* 6, 967-974.

- 5 Wagner, R. W. (1994): Gene inhibition using anti-sense oligodeoxynucleotides, *Nature*, 372, 333-335.

CLAIMS:

1. A formulation for use in therapeutic and/or cosmetic treatment, which formulation comprises:

5

at least one anti-sense oligodeoxynucleotide to a connexin protein; and

a sustained release vehicle for said oligodeoxynucleotides.

10 2. A formulation according to claim 1 which contains oligodeoxynucleotides to one connexin protein only.

3. A formulation according to claim 2 wherein said connexin protein is connexin 43, connexin 26, connexin 31.1 or connexin 32.

15

4. A formulation according to claim 1 which contains oligodeoxynucleotides to more than one connexin protein.

5. A formulation according to claim 4 in which one of the connexin proteins to which oligodeoxynucleotides are directed is connexin 43.

20

6. A formulation according to claim 4 which includes oligodeoxynucleotides directed to at least two of connexin 26, connexin 31.1, connexin 32 and connexin 43.

25

7. A formulation according to claim 3, claim 5 or claim 6 in which the oligodeoxynucleotide to connexin 43 is selected from:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC;

30

GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC; and

GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT.

8. A formulation according to claim 3 or claim 6 in which the oligodeoxynucleotide to connexin 26 is:

35

TCC TGA GCA ATA CCT AAC GAA CAA ATA.

9. A formulation according to claim 3 or claim 6 in which the oligodeoxynucleotide to connexin 31.1 is:

5 CGT CCG AGC CCA GAA AGA TGA GGT C.

10. A formulation according to claim 3 or claim 6 in which the oligodeoxynucleotide to connexin 32 is:

10 TTT CTT TTC TAT GTG CTG TTG GTG A.

11. A formulation according to any preceding claim in which the sustained release vehicle is, or includes, a gel.

15 12. A formulation according to claim 11 in which the gel is a Pluronic gel.

13. A formulation according to any preceding claim which further includes a surfactant to assist with oligodeoxynucleotide penetration into cells.

20 14. A method of site-specific downregulation of connexin protein expression for a therapeutic and/or cosmetic purpose which comprises administering a formulation as defined in any one of claims 1 to 13 to a site on or within a patient at which said downregulation is required.

25 15. A method of reducing neuronal cell death which would otherwise result from a neuronal insult to a specific site in the brain, spinal cord or optic nerve of a patient which comprises the step of administering a formulation as defined in any one of claims 1 to 13 to said site to downregulate expression of connexin protein(s) at and immediately adjacent said site.

30

16. A method according to claim 15 in which the formulation is administered to reduce neuronal loss due to physical trauma to the brain, spinal cord or optic nerve.

17. A method according to claim 15 or claim 16 in which the formulation is
35 administered in a sufficient amount to downregulate expression of said connexin protein(s) for at least 24 hours post-administration.

18. A method of promoting wound healing in a patient which comprises the step of administering a formulation as defined in any of claims 1 to 13 to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

19. A method according to claim 18 in which the wound is the result of trauma.

20. A method according to claim 19 in which the trauma is a burn.

21. A method according to claim 18 in which the wound is the result of surgery.

22. A method of reducing inflammation as part of treating a wound and/or tissue subjected to physical trauma which comprises the step of administering a formulation as defined in any one of claims 1 to 13 to, or proximate to, said wound or tissue.

23. A method according to claim 22 in which the formulation is administered to reduce inflammation due to physical trauma to the brain, spinal cord or optic nerve.

24. A method of decreasing scar formation in a patient who has suffered a wound which comprises the step of administering a formulation as defined in any one of claims 1 to 13 to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

25. A method of skin rejuvenation or thickening for a cosmetic or therapeutic purpose which comprises the step of administering, once or repeatedly, a formulation as defined in any one of claims 1 to 13 to the skin surface.

26. A method according to claim 25 wherein said formulation includes oligodeoxynucleotides directed to connexin 43 and is administered to regulate epithelial basal cell division and growth.

27. A method according to claim 25 wherein said formulation includes oligodeoxynucleotides directed to connexin 31.1 and is administered to regulate outer layer keratinisation.

28. A method according to any one of claims 25 to 27 wherein the formulation is a cream.
- 5 29. The use of at least one anti-sense oligodeoxynucleotide to a connexin protein in the manufacture of a medicament for downregulating expression of said connexin protein for a therapeutic or cosmetic purpose.
- 10 30. The use of claim 29 wherein said medicament is for reducing neuronal cell death which would otherwise result from a neuronal insult.
31. The use of claim 29 wherein said medicament is for promoting wound healing.
- 15 32. The use of claim 29 wherein said medicament is for reducing inflammation.
33. The use of claim 29 wherein said medicament is for decreasing scar formation.
- 20 34. The use of claim 29 wherein said medicament is for skin rejuvenation for a cosmetic or therapeutic purpose.

WEST-WALKER BENNETT

per *S. Zell*

ATTORNEYS FOR THE APPLICANT

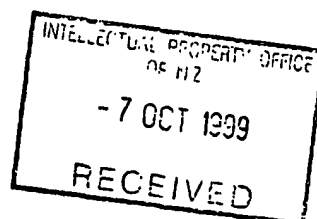


FIGURE 1A

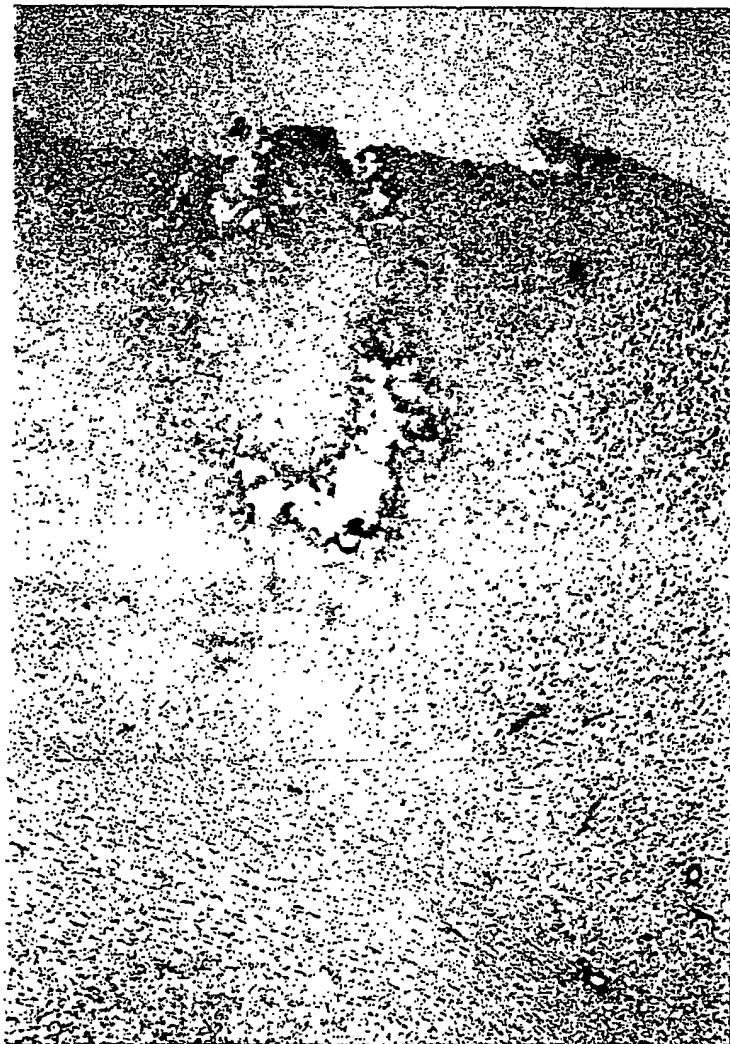


FIGURE 1B



FIGURE 1C



FIGURE 1D



FIGURE 2A



FIGURE 2B



FIGURE 3A



FIGURE 3B



FIGURE 4A

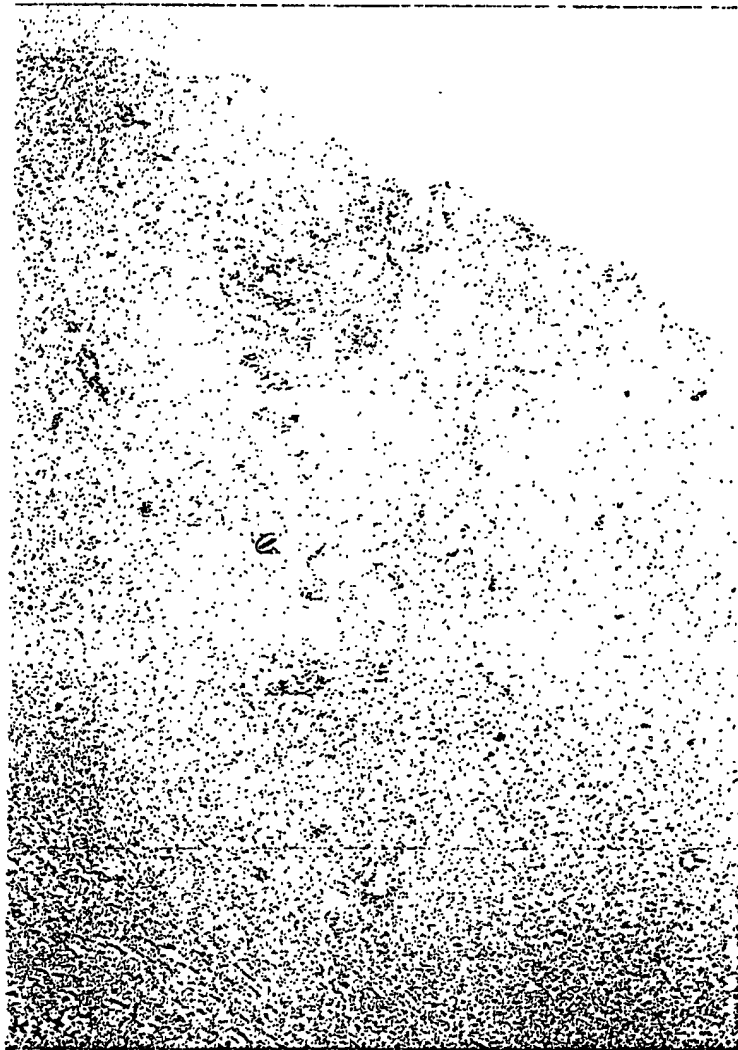


FIGURE 4B



FIGURE 5

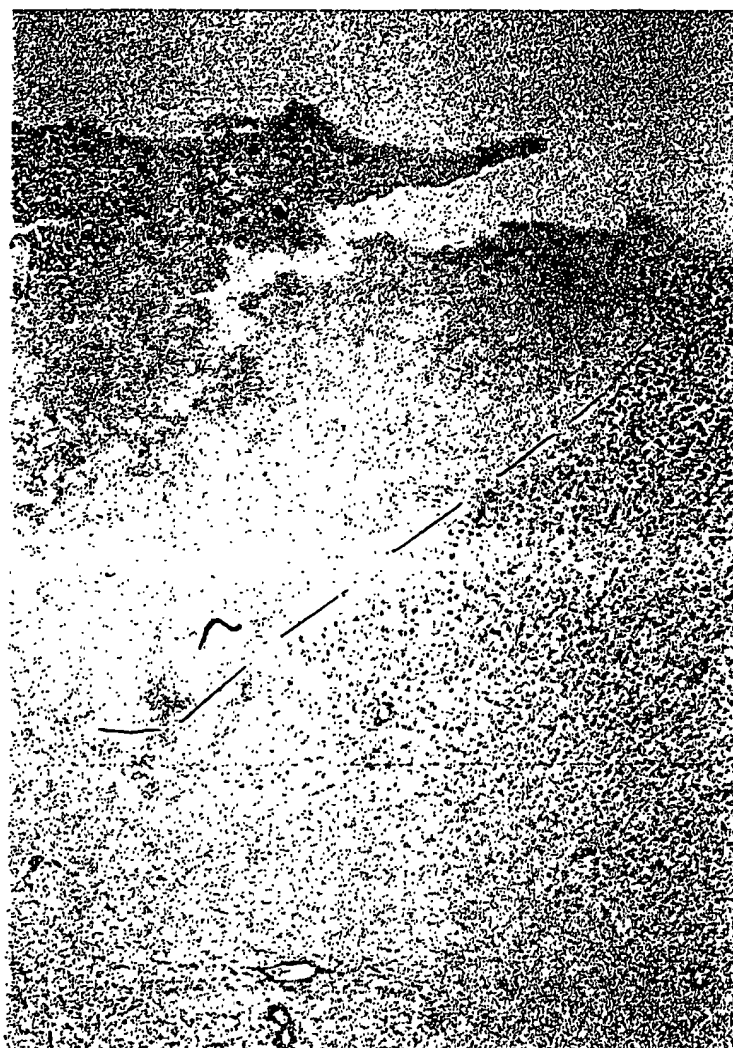


FIGURE 6

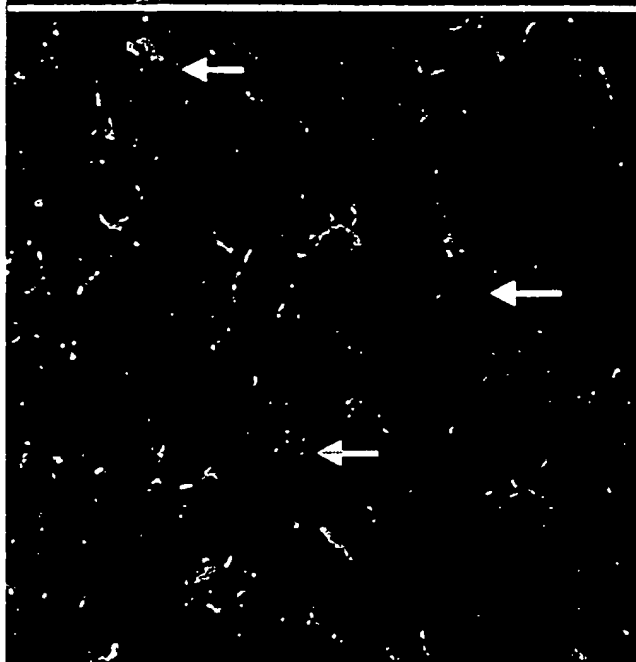
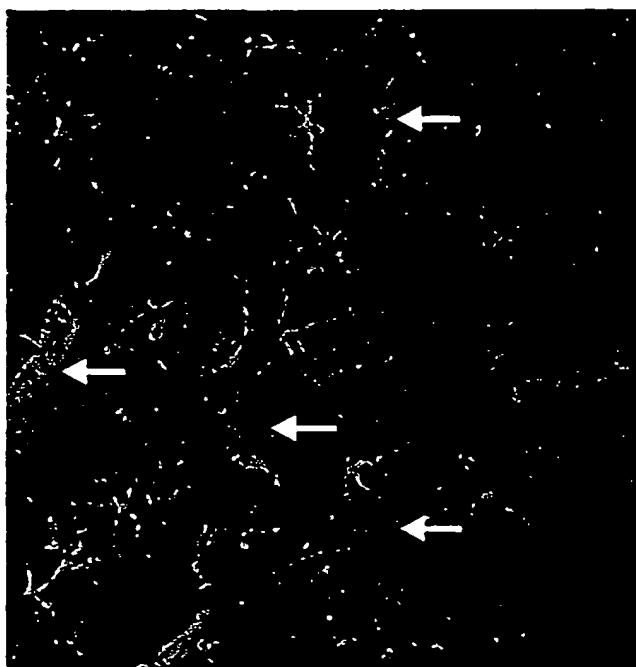


FIGURE 7

Comparison of lesion areas with different treatments

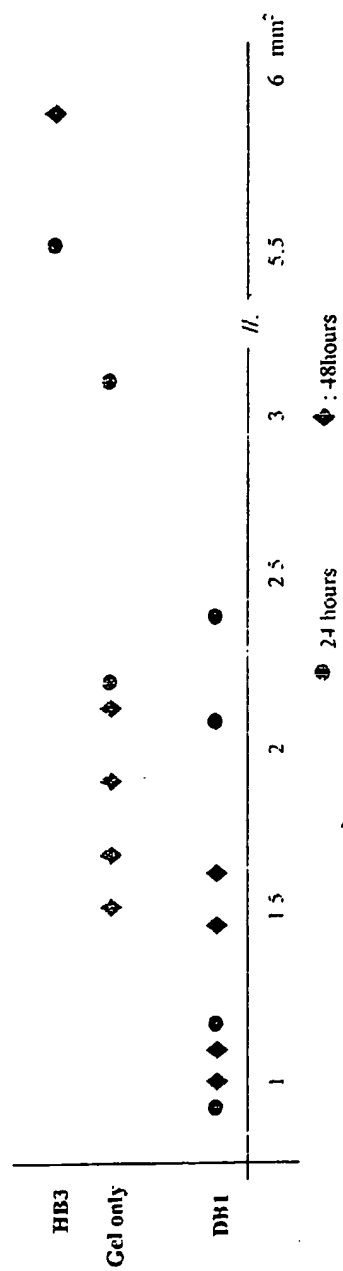


FIGURE 8

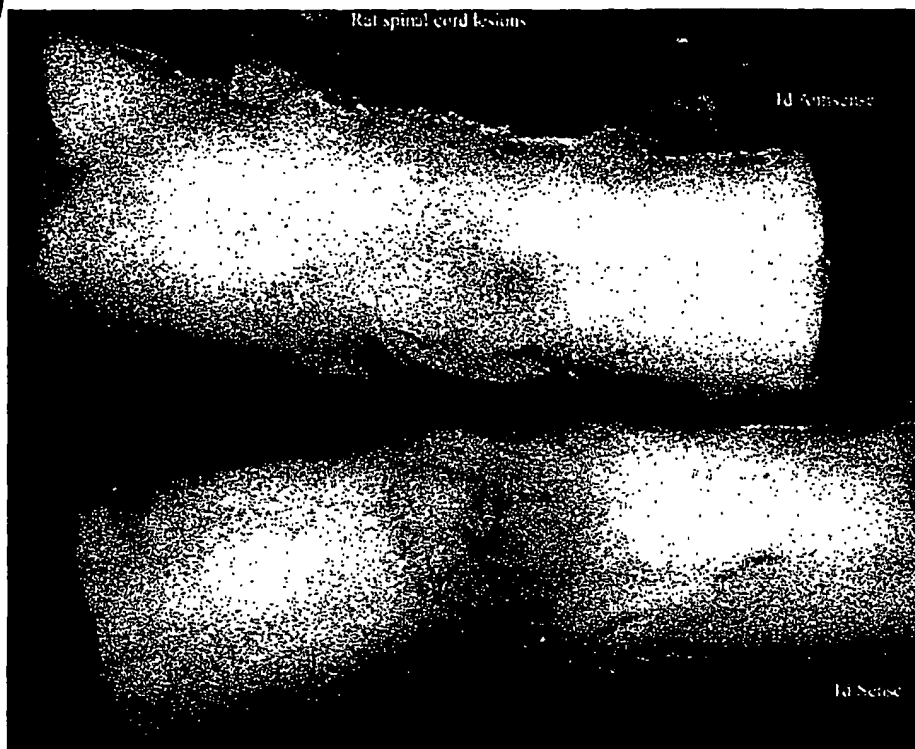
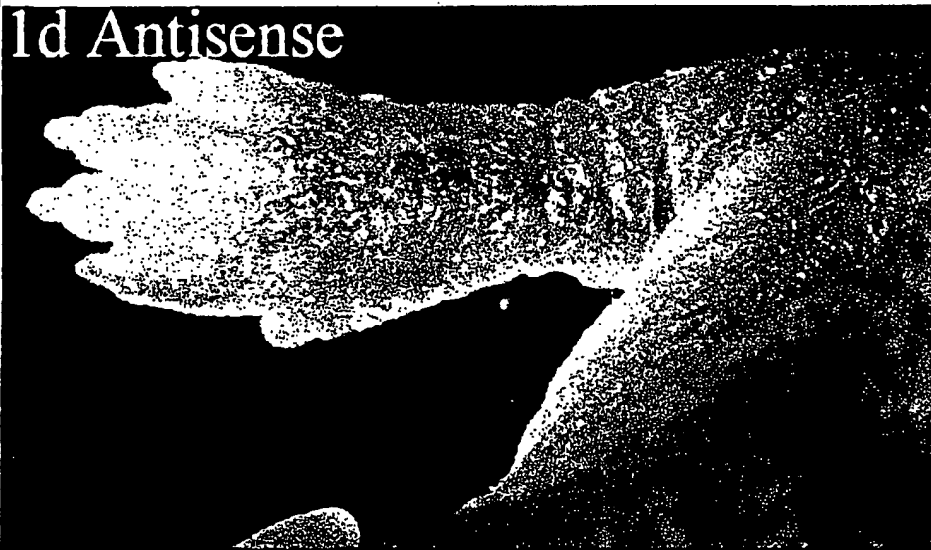


FIGURE 9

1d Antisense



1d Sense

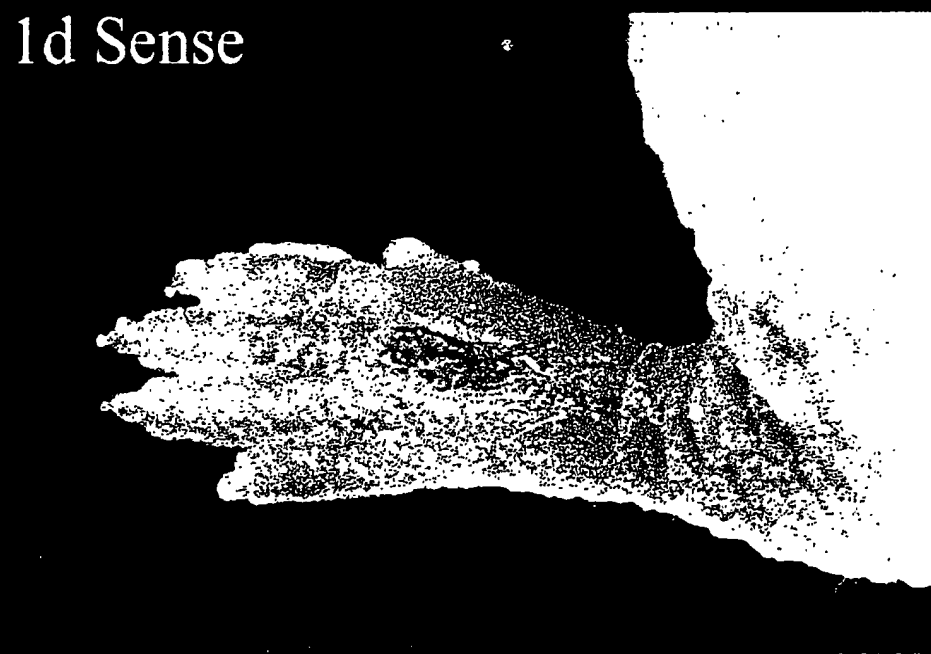


FIGURE 10

Antisense 1d



Sense 1d

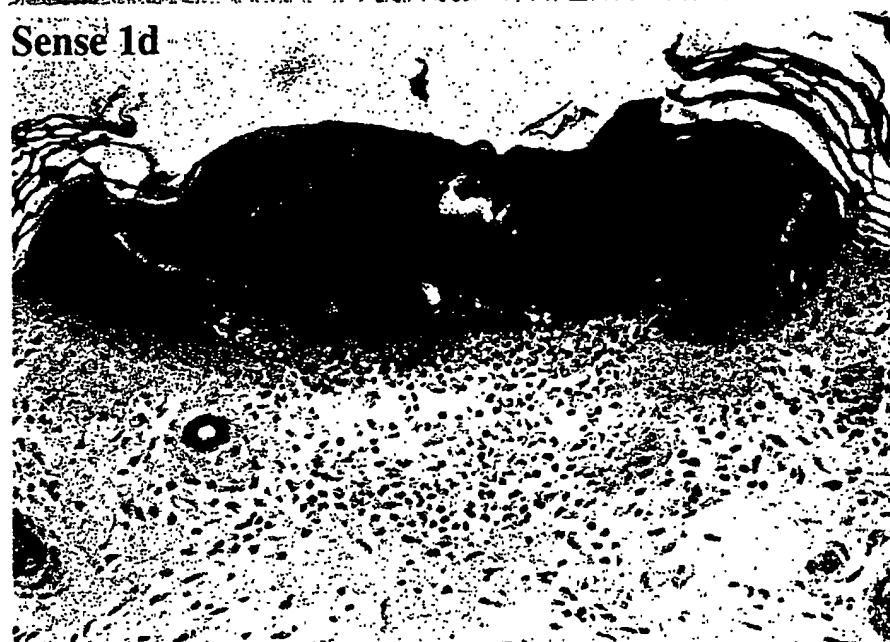


FIGURE 11

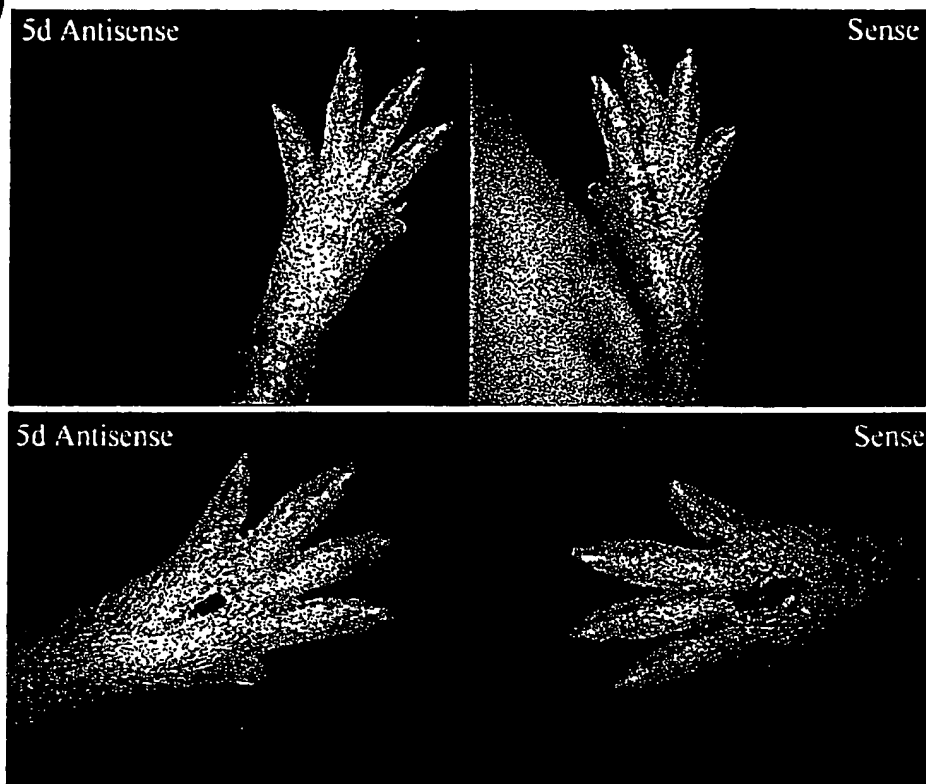


FIGURE 12



FIGURE 13

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.